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Groucho-Associated Transcriptional Repressor Ripply1 Is Required for Proper Transition from the Presomitic Mesoderm to Somites

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Summary

Concomitant with the transition from the presomitic mesoderm (PSM) to somites, the periodical gene expression characteristic of the PSM is drastically changed and translated into the segmental structure. However, the molecular mechanism underlying this transition has remained obscure. Here, we show that *ripply1*, encoding a nuclear protein associated with the transcriptional corepressor Groucho, is required for this transition. Zebrafish *ripply1* is expressed in the anterior PSM and in several newly formed somites. Ripply1 represses *mesp-b* expression in the PSM through a Groucho-interacting motif. In *ripply1*-deficient embryos, somite boundaries do not form, the characteristic gene expression in the PSM is not properly terminated, and the initially established rostrocaudal polarity in the segmental unit is not maintained, whereas paraxial mesoderm cells become differentiated. Thus, *ripply1* plays dual roles in the transition from the PSM to somites: termination of the segmentation program in the PSM and maintenance of the rostrocaudal polarity.

Introduction

In vertebrates, the basis of the segmental pattern in the trunk and tail is established during somitogenesis. Somites, which are transient epithelial spheres of paraxial mesoderm cells, give rise to metameric structures, such as vertebrae and ribs, and impose segmental patterns on the vascular and peripheral nervous systems. Somites are synchronously generated from the anterior end of the unsegmented mesenchymal precursor tissue, called the presomitic mesoderm (PSM), in an anterior to posterior direction in a rhythmic fashion at regular spatiotemporal intervals.

Prior to morphological segmentation, which is a process including intersomitic boundary formation and mesenchymal-epithelial transition, a segmental prepattern, characterized by segmental gene expression, is established in the anterior PSM (Pourquie, 2003; Rida et al., 2004; Saga and Takeda, 2001). For instance, expression of mouse *Mesp2*, a bHLH transcription factor, is initially expressed in a segment-wide domain in the anterior PSM (Saga et al., 1997; Saga and Takeda, 2001). This initial segmental expression domain is defined by a molecular oscillator, referred to as the "segmentation clock," which is composed of the Notch signaling pathway and *hairy/Enhancer of split*-related transcription factors (Holley and Takeda, 2002; Maroto and Pourquie, 2001) and is permitted to "tick" by FGF and Wnt signaling in the posterior PSM (Galceran et al., 2004; Hofmann et al., 2004; Kawamura et al., 2005). Subsequent to the initial establishment of a presumptive segmental unit, *Mesp2* expression becomes localized in the rostral half of a presumptive segmental unit. In mice, this localized Mesp2 represses Notch signaling activity through lunatic fringe, resulting in rostrocaudal compartmentalization of the segmental unit (Saga and Takeda, 2001; Takahashi et al., 2000). In addition to these molecules, some permissive factors, including the T-box transcription factor *fused somites (fss)/tbx24* (Nikaido et al., 2002; van Eeden et al., 1996), and *Foxc1* and *c2*, a winged helix transcription factor, are also required for the progression of segmental patterning in the anterior PSM (Kume et al., 2001; Topczewska et al., 2001). Thus, the establishment of the segmental prepattern in the anterior PSM has been revealed to require a number of processes regulated by many transcription factors and signaling molecules.

Then, concomitant with the transition from the anterior PSM to somites, the characteristic gene expression in the PSM is translated into the segmental structure. A well-characterized process during or after this transition is the formation of the intersomitic boundary, which appears to be triggered by modulation of Notch activity (Sato et al., 2002) and also requires repulsive interaction between Ephrins and EphA4 and the subsequent accumulation of fibronectin (Barrios et al., 2003; Durbin et al., 1998; Ju Lich et al., 2005; Koshida et al., 2005). However, most of the other events accompanying the transition from the anterior PSM to somites have remained obscure. For instance, it is uncertain whether specific molecules are required for maintenance of the initially established segmental prepattern and, if so, how this prepattern is maintained during somite development. Furthermore, although gene expression characteristic of the segmental patterning process in the PSM is drastically changed when this transition takes place, the molecular mechanism, as well as the biological significance, of the termination of the PSM-specific gene expression has remained totally unclear.

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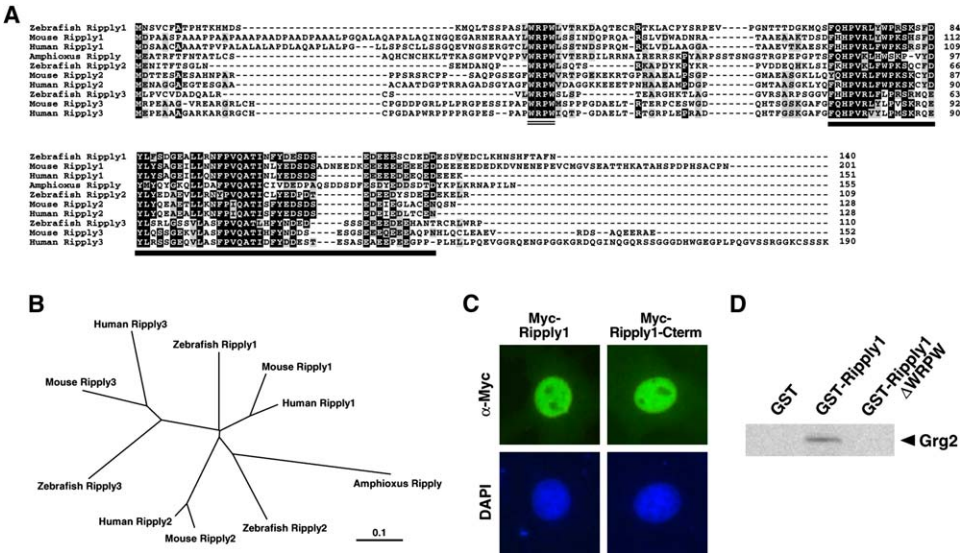

Figure 1. *ripply1* Encodes a Nuclear Protein that Interacts with the Transcriptional Corepressor Groucho

(A) Comparison of Ripply proteins in zebrafish, mouse, human, and amphioxus. Gapped alignments were made by the ClustalX program. Identical residues are shown on a black background, and similar residues are shown on a gray background. Numbers to the right of the sequences indicate the position of the right-most amino acid. The conserved WRPW tetrapeptide is underscored with a double underline, and the Ripply homology domain is underscored with a thick black line. Nucleotide and amino acid sequences are registered with the following accession numbers: zebrafish *ripply1*, AB212219; zebrafish *ripply2*, AB212220; zebrafish *ripply3*, AB212221; mouse *Ripply1*, AB212222; mouse *Ripply2*, AB212223. The other amino acid sequences were derived from the database: human *Ripply1*, BX106639; human *Ripply2*, BE672047; human *Ripply3*, NM018962; mouse *Ripply3*, NM133229; amphioxus *ripply*, AY860953.

(B) A phylogenetic tree of Ripply proteins. The tree was constructed by the Neighbor-Joining method (Saitou and Nei, 1987) with the sequences of the Ripply homology domain.

(C) Subcellular localization of the Myc-tagged Ripply1 protein. Myc-tagged Ripply1 proteins were introduced into Cos7 cells and were visualized with Alexa488-conjugated antibody. Nuclei were counterstained with DAPI.

(D) The WRPW motif-dependent interaction of Ripply1 with zebrafish Groucho-related Grg2 proteins in vitro. A pull-down assay was performed with in vitro-labeled Grg2 protein; purified GST, GST-Ripply1, and GST-Ripply1ΔWRPW. GST-Ripply1, not GST alone or GST-Ripply1ΔWRPW, physically associates with Grg2.

To gain insight into the molecular mechanism underlying the segmentation process, we sought to identify genes specifically expressed in the PSM and tailbud by performing an in situ hybridization screening with zebrafish embryos (Kawamura et al., 2005). In the process of this screening, we found a gene, designated *ripply1*, encoding a protein with no significant structural similarity to any previously reported proteins. The result of a knockdown experiment with zebrafish embryos indicated that *ripply1* is essential for intersomitic boundary formation. Strikingly, precise analysis with a number of molecular markers revealed that *ripply1* played essential roles in two different processes during the transition from the PSM to somites, i.e., termination of gene expression characteristic of segmental patterning in the PSM and maintenance of the rostrocaudal polarity of somites. As the significance and molecular mechanism of these two processes have remained totally unknown, functional analysis of *ripply1* should further increase our understanding of the molecular mechanism of somitogenesis.

Results

ripply1 Represents a Gene Family Structurally Conserved in Vertebrates and Cephalochordates

By in situ hybridization screening of mRNA expressed in the PSM or tailbud region of zebrafish embryos at the 18- to 21-somite stages (Kawamura et al., 2005), we identified a gene specifically expressed in the anterior PSM and several newly formed somites. This gene encodes a putative protein of 140 amino acids, and a BLAST analysis revealed that at least two other structural homologs exist in zebrafish (Figure 1A). Counterparts to each of these three structurally related genes were also found to exist in human and mouse genomes. One of the human counterparts had also been identified as one of the genes in the Down's syndrome critical region (*ripply3*, see below; Shibuya et al., 2000). In addition, a structurally related gene had been identified in many other vertebrates as well as in Amphioxus, in which somites are similarly generated as they are in vertebrates (Figure 1B); however, no related gene was found in other invertebrates, including *C. elegans* and *Drosophila*. Since expression of the gene identified by our in situ hybridization screening was evident in the anterior PSM and gradually decreased in a posterior-to-anterior direction in somites like a ripple (Figure 2D), we designated this gene as *ripply1* and the other two related genes as *ripply2* and *ripply3*.

Comparison of the amino acid sequences among Ripply proteins showed that the WRPW tetrapeptide, which is found at the carboxyl terminus of Hairy/Enhancer of split-related proteins and is known to recruit the transcriptional corepressor Groucho protein (Fisher et al., 1996; Paroush et al., 1994), was completely conserved

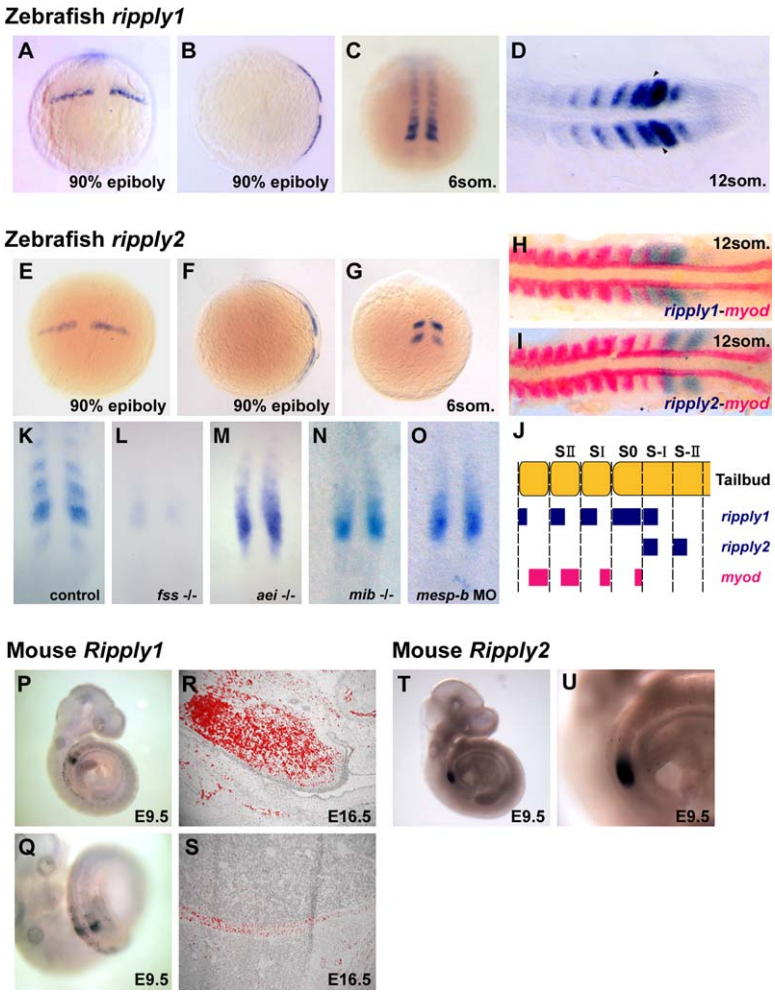

Figure 2. Expression Patterns of *ripply* Genes in Zebrafish and Mouse

(A–D) Expression patterns of zebrafish *ripply1* during embryogenesis. (A) Dorsal view at 90% epiboly. First, expression of *ripply1* mRNA is detected in a stripe along the notochord. (B) Animal pole view of the same embryos shown in (A). The signal is observed in the inner layer, i.e., the mesoderm. (C) Dorsal view at the 6-somite stage. A total of 5–6 pairs of stripes are observed in the somites and PSM. (D) Flat-mounted embryo at the 12-somite stage. The newly formed somite boundary is emphasized by the arrowhead. Zebrafish *ripply1* mRNA is strongly expressed in the anterior end of the unsegmented PSM and is polarized in the anterior region of somites.

(E–G) Expression patterns of *ripply2* mRNA in zebrafish embryos. (E) Dorsal view at 90% epiboly. *ripply2* mRNA is first expressed in the paraxial mesoderm. (F) Animal pole view of the same embryos shown in (E). (G) The 6-somite stage. Two pairs of stripes are detected in the PSM.

(H–J) Precise expression analysis of *ripply1* and *ripply2* in the PSM. Two-color in situ hybridization was performed by using probes for *ripply1* (blue) and *myod* (red, [H]) and probes for *ripply2* (blue) and *myod* (red, [I]). (J) Schematic drawing of expression domains of *ripply1* and *ripply2*. The nomenclature for segmented and prospective somites is according to Pourquie and Tam (2001).

(K–O) The expression of *ripply1* is disturbed in embryos deficient for somite segmentation. Segmental expression of *ripply1* mRNA is observed in (K) control embryos, whereas it is significantly reduced in (L) *fss* mutants (n = 12; 100% affected). In addition, the expression of *ripply1* mRNA is scattered in (M) *aei/deltaD* mutants (n = 12; 100% affected), in (N) *mib* mutants (n = 8; 100% affected), and in (O) *mesp-b* MO-injected embryos (n = 10; 100% affected). Embryos were fixed at the 10- to 12-somite stages, and they were stained by using a *ripply1* probe. Evidence to indicate that injection of the *mesp-b* MO caused the expected segmentation defect is shown in Figure S1.

(P–S) Expression patterns of mouse *Ripply1*. (P and Q) Signals are observed in the anterior PSM and somites of E9.5 embryos. [^{35}S]-labeled antisense RNA probe of *Ripply1* was hybridized to sagittal sections of mouse E16.5 embryos. Signals (red) are detected in the (R) tongue and (S) diaphragm.

(T and U) Expression patterns of mouse *Ripply2*. The signal is detected in the anterior PSM of mouse embryos.

near their amino termini (Figure 1A). In addition, an ~50 amino acid length sequence at the carboxyl terminus, which we termed the Ripply homology domain, was also conserved but did not show any significant similarity with other motifs previously identified. These results indicate that *ripply* genes encode proteins representing a gene family with unique structural characteristics conserved in both vertebrates and cephalochordates.

The existence of this WRPW motif suggests that Ripply is a nuclear protein that interacts with the transcriptional corepressor Groucho, although no typical nuclear localization signal was found in the amino acid sequences of Ripply proteins. To investigate the subcellular localization of the Ripply protein, we examined the localization of myc-tagged Ripply1 protein in Cos7 cells. Signals specific to anti-myc antibody were detected predominantly in Cos7 cell nuclei (Figure 1C). Similarly, myc-tagged Ripply1 lacking the WRPW motif (Myc-Ripply1ΔWRPW) or the N-terminal region up to the WRPW motif (Myc-Ripply1-Cterm) retained the property of nuclear localization. Therefore, Ripply1 is a nuclear protein, and its C-terminal region to the WRPW motif is sufficient for the nuclear localization. Then, to examine whether Ripply1 could bind to Groucho, we performed in vitro pull-down assays. GST-fused Ripply1, but not a control GST protein, bound to radiolabeled zebrafish Grg2 (Figure 1D), a member of the zebrafish Groucho-related family of proteins (Wulbeck and Campos-Ortega, 1997). This binding was dependent on the WRPW motif, because Ripply1 lacking the WRPW motif did not associate efficiently with Grg2. These results indicate that Ripply1 functions in the nucleus in concert with the transcriptional corepressor Groucho.

ripply Genes Exhibit Characteristic Expression in the PSM and Somites of Zebrafish and Mouse Embryos

To investigate the spatiotemporal expression of *ripply* genes during embryogenesis, we examined their expression in zebrafish and mouse embryos. In zebrafish, *ripply1* was first expressed in a pair of stripes in the

paraxial mesoderm at the end of gastrulation (Figures 2A and 2B). During the segmentation period, *ripply1* transcripts were seen in 6–7 pairs of stripes in the PSM and somites (Figure 2C). As the segmentation proceeded, the expression domain relatively moved toward the posterior but was still confined to the PSM and somites (Figure 2D). Comparison of the expression domain of *ripply1* with that of *myod*, which is expressed in the caudal region of each segmented and prospective somite (Weinberg et al., 1996), showed that, during the process of somitogenesis, *ripply1* mRNA was first expressed at S-I, a prospective segmental unit in the PSM previously defined by Pourquie and Tam (2001); was expressed at its highest level in the segment-wide domain at S0, a prospective segment unit between S-I and the most newly formed somite; and subsequently became localized in the rostral compartment of somites (Figures 2D, 2H, and 2J). On the other hand, the expression of *ripply2* was first observed in the paraxial mesoderm at the 90% epiboly stage, as in the case of *ripply1*, and it was subsequently confined to the PSM (Figures 2E, 2F, and 2G). Comparing the expression pattern of *ripply2* with that of *myod* revealed the presence of the former's signal in the rostral compartment of S-I and S-II, a prospective segmental unit posterior to S-I (Figures 2I and 2J). In contrast, we did not detect any specific expression of *ripply3* in zebrafish embryos at these early stages (data not shown).

Characteristic expression of *Ripply1* and *Ripply2*, was also observed in mouse embryos. Mouse *Ripply1* expression was strong in S0 and remained weak in the anterior parts of several newly formed somites at E9.5–E10.5 (Figures 2P and 2Q). On the other hand, mouse *Ripply2* was first expressed in S-I at E8.5 (Figures 2T and 2U). This expression pattern of *Ripply2* was basically maintained until E13.5, but an additional stripe of its expression was sometimes observed at the rostral part of S0. Mouse *Ripply1* was also expressed in tongue, diaphragm, and intercostal muscles at E16.5 (Figures 2R and 2S; data not shown), whereas no specific signal of *Ripply2* mRNA was detected at this stage (data not shown). Therefore, *ripply1* and *ripply2* are commonly expressed in the PSM of both zebrafish and mice. This similarity in expression suggests that the roles of these two genes may have been conserved during the evolution of vertebrates.

The Expression of *ripply1* Is Disturbed in Embryos Deficient for Somite Segmentation

As a first step to reveal the roles of *ripply* genes in development, we focused on zebrafish *ripply1*. Since the expression of this gene started from the anterior PSM during somitogenesis, we examined the effect of a couple of genes involved in the development of the anterior PSM on the expression of *ripply1*. In *fss/tbx24* homozygous mutant embryos, the level of *ripply1* mRNA was significantly reduced (Figures 2K and 2L), indicating that the expression of *ripply1* required *fss/tbx24.* On the other hand, *ripply1* was expressed in a scattered pattern in the anterior PSM and somites in embryos homozygous for mutant *aei/deltaD* or *mind bomb*, the latter of which encodes a ubiquitin ligase required for Delta-Notch signaling (Figures 2M and 2N) (Holley et al., 2000; Itoh et al., 2003), as well as in embryos treated with DAPT, a γ-secretase inhibitor (data not shown). In addition, embryos injected with *mesp-b*-specific antisense morpholino oligonucleotide (MO) also exhibited a scattered expression of *ripply1* (Figure 2O). Thus, Notch signaling and *mesp-b* were not required for the induction of *ripply1* expression, but rather for the proper patterning of its expression.

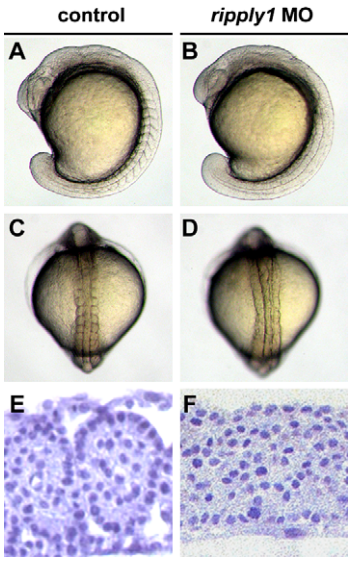

Figure 3. Injection of *ripply1* MO Results in the Absence of All of the Somite Boundaries

(A–D) In the *ripply1* MO-injected embryos, the somites are completely fused from the (B and D) first somite, whereas no apparent segmental defects are observed in the (A and C) 5mis-*ripply1* MO-injected embryo. (A and B) Lateral views and (C and D) dorsal views at the 12-somite stage are shown. *ripply1* and 5mis-*ripply1* MOs were injected at a concentration of 1.0 μg/μl.

(E and F) Parasagittal sections of (E) control and (F) *ripply1* MO-injected embryos. The sections were briefly stained with hematoxylin.

Knockdown of *ripply1* Results in the Complete Absence of Somite Boundaries

To investigate the function of *ripply1* during somitogenesis, we next performed MO-mediated gene knockdown experiments with zebrafish embryos. Injection with either of two independent MOs specific for *ripply1* resulted in the complete absence of all of the somite boundaries, whereas that of their 5 base-substituted control MOs caused no such defect (Figure 3 and Table S1; see the Supplemental Data available with this article online). The specific interference by these MOs was further confirmed by the finding that in vitro synthesis of Ripply1 protein was inhibited with the *ripply1* MO1, but not the 5mis-*ripply* MO1 (Supplemental Data). Thus, *ripply1* is required for the proper progression of morphological segmentation of somites. On the other hand, injection of *ripply 2*-specific MOs did not cause any obvious defect.

ripply1 Is Required for Termination of Gene Expression Characteristic of the Process of Segmental Patterning in the PSM

To investigate further the disturbance of somite segmentation in *ripply1* MO-injected embryos, we examined the expression patterns of a series of genes characteristically expressed during the segmentation process

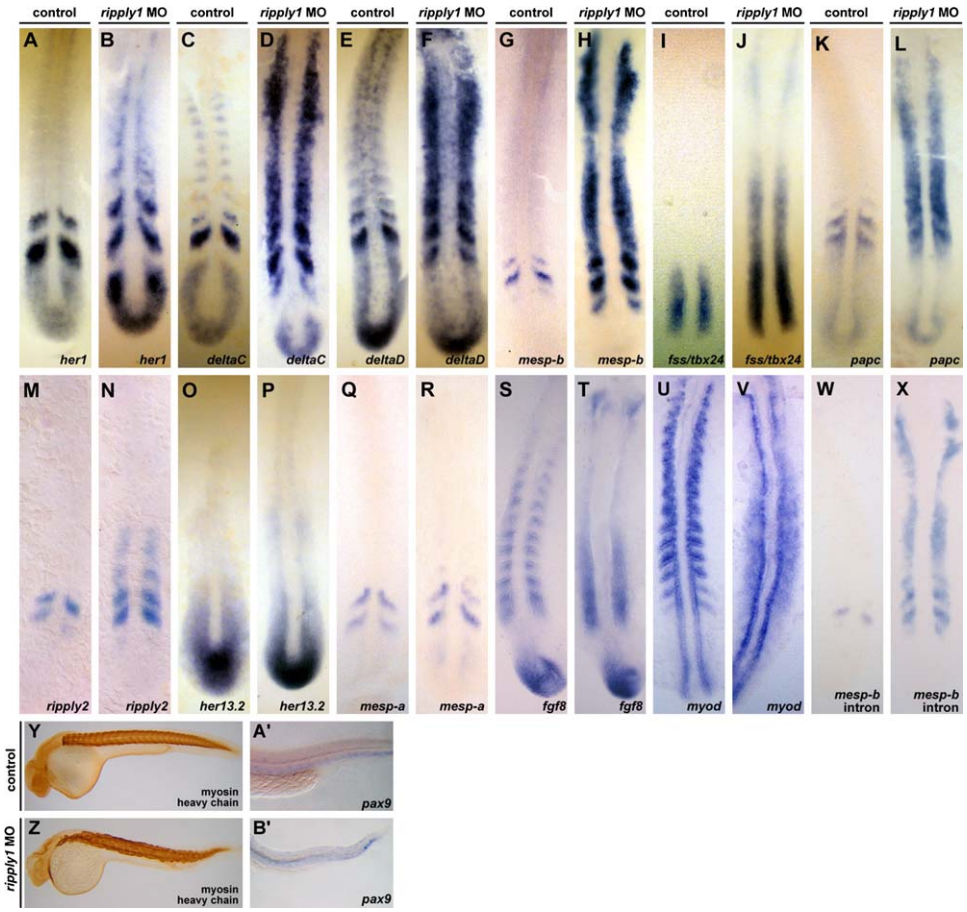

Figure 4. Abnormal Expression of Genes Involved in the Segmental Patterning in *ripply1*-Deficient Embryos

(A–N) Expression of genes that are expressed in the anterior PSM and known to be involved in somite segmentation, such as (A and B) *her1* (n = 25; 100% affected), (C and D) *deltaC* (n = 24; 100% affected), (E and F) *deltaD* (n = 25; 100% affected), (G and H) *mesp-b* (n = 25; 100% affected), (I and J) *fss/tbx24* (n = 24; 100% affected), (K and L) *papc* (n = 25; 100% affected), and (M and N) *ripply2* (n = 10; 100% affected), is expanded anteriorly in the paraxial mesoderm in *ripply1*-deficient embryos.

(O and P) *her13.2*, normally expressed in the posterior PSM, is not significantly altered in *ripply1*-deficient embryos (n = 25; 0% affected).

(Q and R) Segmental expression of *mesp-a* appears indistinguishable between (Q) control and (R) *ripply1* MO-injected embryos (n = 25; 0% affected).

(S–V) Mixed anteroposterior polarity of somites is also seen in *ripply1*-deficient embryos. Expression of (S and T) *fgf8*, which is normally confined to the anterior region of somites, is uniformly detected (n = 25; 100% affected), and that of (U and V) *myod*, which is normally expressed in the posterior region of somites, is also uniformly observed in *ripply1* MO-injected embryos (n = 24; 100% affected).

(W and X) Transcriptionally active state of *mesp-b* in *ripply1* morphants. In situ hybridization was carried out by using a *mesp-b* intron probe. (W) Segmental expression of *mesp-b* nascent transcripts is observed in the PSM of the control embryo at the 12-somite stage. (X) In contrast, the primary *mesp-b* mRNA was ectopically detected in the somites of *ripply1* MO-injected embryos, as found with the *mesp-b* exon probe (n = 15; 100% affected). Embryos in (A)–(V) were fixed at the 10- to 12-somite stages, flat-mounted, and are shown from the dorsal aspect.

(Y–B′) (Y and Z) Myosin heavy chain protein (n = 10; 0% affected), involved in myogenesis, and (A′ and B′) *pax9* (n = 10; 0% affected), related to the sclerotome, are expressed in *ripply1* MO-injected embryos at 36 hr postfertilization.

at the 10- to 12-somite stages. First, the expression of a *hairy/Enhancer of split*-related gene, *her1*, and a gene encoding Notch ligand, *deltaC*, exhibited a cyclic wave of expression patterns in the PSM of *ripply1*-deficient embryos similar to that seen in control embryos (Figures 4A–4D) (Holley et al., 2000; Jiang et al., 2000; Sawada et al., 2000). In addition, in the posterior PSM, *her13.2*, another *her* gene, one required for oscillating expression of *her1* (Kawamura et al., 2005), was also expressed in a similar manner as in the control embryos (Figures 4O and 4P). Thus, the segmentation clock appeared to function normally in the PSM of *ripply1*-deficient embryos.

Strikingly, in *ripply1*-deficient embryos, the characteristic gene expression in the anterior PSM was not properly terminated. Accompanying the transition from the anterior PSM to somites in normal embryos, the expression patterns of genes involved in the segmentation machinery are drastically changed. For instance, the expression of several genes, including *her1* and *mesp-a* and *-b*, is abruptly terminated at this transition, whereas that of other genes, including *deltaC* and *deltaD*, *fss/tbx24*, and *paraxial protocadherin* (*papc*), remains in somites, but their expression patterns are drastically changed at this transition (Durbin et al., 2000; Holley et al., 2000; Jiang et al., 2000; Nikaido et al., 2002; Sawada et al., 2000; Yamamoto et al., 1998). In *ripply1*-deficient embryos, the expression of *her1* and *mesp-b* was not properly terminated at the level of the anterior

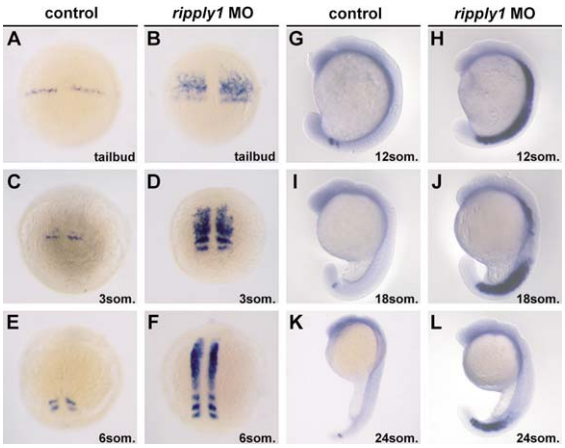

Figure 5. Ectopic Expression of *mesp-b* mRNA in *ripply1*-Deficient Embryos during Embryogenesis

(A–L) (A, C, E, G, I, and K) Control embryos and (B, D, F, H, J, and L) *ripply1* MO-injected embryos in (A–F) dorsal view and (G–L) lateral view are shown. In control embryos, expression of *mesp-b* is first observed in a stripe along the notochord at 90% epiboly and remains confined to the anterior PSM during the segmentation period. In *ripply1* MO-injected embryos, the expression domain of *mesp-b* in the paraxial mesoderm appears sequentially expanded until the 12-somite stage, and its signal ceases concomitant with the end of somite segmentation.

end of the PSM, but it continued ectopically to the axial level, where somites should be formed in normal embryos (Figures 4A, 4B, 4G, and 4H). Similarly, defective termination of the expression in the PSM-specific patterns was observed in the expression of *deltaC* and *deltaD*, *fss/tbx24*, *papc*, and *ripply2* (Figures 4C–4F and 4I–4N). The ectopic expression of these genes occurred throughout the period of somite segmentation. For instance, the ectopic expression of *mesp-b* was already evident at the tailbud stage, and it ceased at the end of segmentation (Figure 5). In contrast to the expression of these genes, that of *mesp-a*, which is also segmental in the anterior PSM of control embryos (Durbin et al., 2000; Sawada et al., 2000), was not altered in *ripply1*-deficient embryos (Figures 4O and 4P). Thus, the characteristic expression of many, but not all, genes involved in the segment patterning process was not properly terminated in *ripply1*-deficient embryos at the transition from the anterior PSM to somites.

To see whether this abnormal termination was caused by defective suppression of transcription or by increased stabilization of mRNAs, we next examined the distribution of *mesp-b* nascent transcripts by using an intron probe. Whereas segmental expression was detected in the anterior PSM of control embryos (Figure 4W), the signal was ectopically detected at the level anterior to the PSM of *ripply1*-deficient embryos as in the case of its exon probe (Figure 4X). Similarly, ectopic expression of de novo transcripts of *her1* was also seen in these embryos by using a *her1* intron probe (data not shown). Therefore, at the transition from the anterior PSM to somites, *ripply1* played an essential role in the repression of the segmental patterning genes at the transcriptional level.

ripply1 Is Required for the Termination of PSM-Specific Gene Expression in a Cell-Autonomous Manner

To address whether *ripply1* is involved in this transcriptional repression in a cell-autonomous or nonautonomous manner, we next transplanted *ripply1*-depleted blastomeres into wild-type embryos and examined the *mesp-b* expression pattern at the 18-somite stage (Figure 6A). In embryos containing wild-type donor cells, *mesp-b* mRNA was specifically expressed in donor cells located in the anterior PSM (data not shown; n = 5, "n" represents the number of embryos that possessed successfully transplanted donor cells in their somites). In contrast, in embryos injected with the *ripply1*-depleted donor cells, the ectopic expression of *mesp-b* was still observed in labeled donor cells specifically located in somites, but not in neighboring host cells (Figures 6B–6E; n = 5). Thus, *ripply1* plays an essential role in transcriptional repression of *mesp-b* in a cell-autonomous manner: that is, *ripply1* needs no intercellular interaction for its suppressive effect on *mesp-b* transcription.

ripply1 Is Required for Maintenance of the Rostrocaudal Polarity of Somites

In addition to defective termination of gene expression, the rostrocaudal polarization was also disturbed in

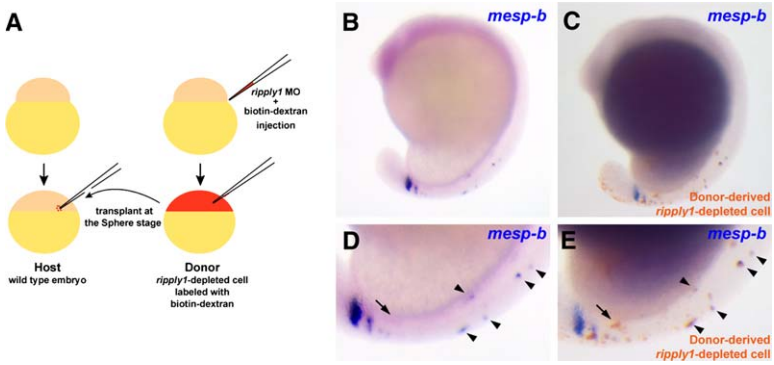

Figure 6. Cell-Autonomous Ectopic Expression of *mesp-b* in the *ripply1*-Depleted Cells Located in the Somites

(A) Schematic representation of the strategy for the transplantation experiments. The donor embryos were prepared by the injection of the *ripply1* MO, biotin-dextran, and rhodamine-dextran at the 1- to 2-cell stage. At the sphere stage, 10–20 blastomeres were sucked from the donor and then transplanted into the margin of wild-type embryos (host) at the same stage. Under a fluorescence microscope, the embryos containing the transplanted *ripply1*-depleted cells in their somites were segregated and subjected to in situ hybridization analysis with the *mesp-b* probe.

(B–E) Ectopic expression of *mesp-b* in a cell-autonomous manner. Dark-blue signals show the expression of *mesp-b*, and brown signals show the *ripply1*-depleted donor cells. (B and D) An embryo stained with the *mesp-b* probe is shown. (C and E) The same embryo is shown after staining the donor cells with anti-biotin antibody. *mesp-b* is normally expressed in the anterior PSM. Ectopic expression of *mesp-b* in the somites is derived from the *ripply1*-depleted donor cells, evidenced by the mergence of dark-blue and brown signals (arrowhead shows a representative cell). In contrast, *mesp-b* is not expressed in the *ripply1*-depleted cells when they are located outside the somites (arrow).

ripply1-deficient embryos. For instance, the expression of *myod*, which is restricted to the caudal region of somites and the presumptive segmental unit in the anterior PSM of normal embryos (Weinberg et al., 1996), was detected uniformly in the anterior PSM and paraxial mesoderm anterior to the PSM of *ripply1*-deficient embryos (Figures 4U and 4V). In addition, *fgf8* and *papc* mRNAs, normally expressed in the rostral part of somites or the presumptive segmental units in the anterior PSM (Reifers et al., 1998; Yamamoto et al., 1998), were observed ubiquitously in *ripply1*-deficient embryos (Figures 4K, 4L, 4S, and 4T). In contrast, in view of the expression pattern of *deltaD*, *mesp-a*, and *mesp-b*, which are normally localized in the rostral compartment of the segmental unit (Durbin et al., 2000; Sawada et al., 2000), the rostrocaudal polarity of the presumptive segmental units in the anterior PSM was partly established (Figures 4E–4H, 4Q, and 4R), although the expression of *deltaD* and *mesp-b* was almost uniformly observed at the level anterior to the PSM. These results indicate that the rostrocaudal polarity in the segmental units is initially established, at least to some extent, but cannot be maintained in *ripply1*-deficient embryos.

Cell Differentiation Proceeds in the Paraxial Mesoderm of *ripply1* MO Embryos

To examine whether defective segmentation of somites may affect the differentiation of paraxial mesoderm cells, we next examined the expression of cell differentiation markers in *ripply1*-deficient embryos. The expression of two differentiation marker genes, *myod*, which is normally expressed in the adaxial cells and in the caudal region of somites (Weinberg et al., 1996), and *pax9*, which is normally expressed in the sclerotome (Nornes et al., 1996), was clearly observed in *ripply1*-deficient embryos (Figures 4U, 4V, 4A′, and 4B′). In addition, immunostaining with anti-myosin heavy chain antibody revealed the presence of differentiated muscle cells in *ripply1*-deficient embryos at 36 hr postfertilization (Figures 4Y and 4Z). Thus, differentiation of paraxial mesoderm cells proceeded in *ripply1*-deficient embryos. That is, the differentiation of paraxial mesoderm cells appears to have proceeded independently of the termination of the characteristic gene expression in the anterior PSM.

Overexpression of *ripply1* Represses *mesp-b* Expression in the Anterior PSM

Our results indicate that *ripply1*, which encodes a nuclear protein associated with the transcriptional repressor Groucho, is required for the proper transcriptional termination of genes involved in somite segmentation, such as *mesp-b* and *her1*, at the transition from the PSM to somite. This observation prompted us to investigate whether *ripply1* itself is able to repress the transcription of genes involved in somite segmentation by introducing *ripply1* mRNA ectopically into zebrafish embryos. Whereas injection of a large amount (100 pg) of synthesized *ripply1* mRNA into embryos at the 1- to 4-cell stage caused a severe reduction in the trunk length, a lower dose (25 pg) of exogenous *ripply1* mRNA resulted in a distinctive segmental disruption of somite boundaries in addition to a slight defect in elongation of the trunk (Figure 7A). In the full-length *ripply1* mRNA-injected embryos showing this segmental defect, the expression level of *mesp-b* was significantly reduced in a WRPW motif-dependent fashion (Figure 7B). In contrast, the expression of *mesp-a*, which was not affected in *ripply1*-deficient embryos, was not significantly altered at the 6-somite stage (Figure 7B). These results indicate that *ripply1* is able to repress *mesp-b* expression, but not that of *mesp-a*, in a manner dependent on the transcriptional corepressor Groucho in the anterior PSM. Thus, *ripply1* appears to function as an essential component of a transcriptional repressor complex underlying the proper transition of the segmentation program from the PSM to somites.

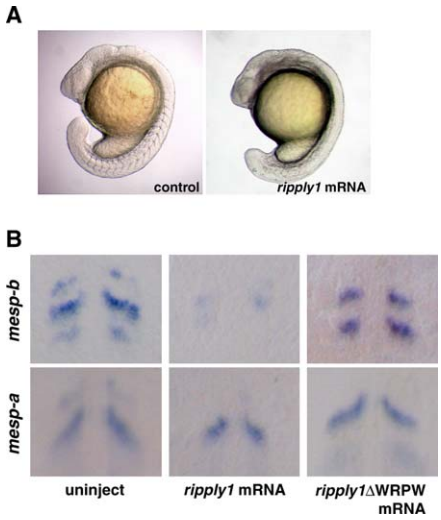

Figure 7. Overexpression of *ripply1* mRNA Represses *mesp-b* Expression in the Anterior PSM

(A) Segmental defect in the *ripply1* mRNA-injected embryos. Synthesized *ripply1* mRNA or control solution was injected into the embryos at the 1- to 4-cell stages.

(B) Decreased expression of *mesp-b* mRNA in the *ripply1* mRNA-injected embryos. Segmental expression of *mesp-b* is decreased in the anterior PSM of *ripply1* mRNA-injected embryos. However, injection of *ripply1*ΔWRPW mRNA does not significantly affect the expression of *mesp-b*. In contrast to that of *mesp-b*, the expression of *mesp-a* was not apparently altered in either *ripply1* or *ripply1*ΔWRPW mRNA-injected embryos.

Discussion

Termination of the Segment Patterning Machinery: A Newly Identified Process in Somite Segmentation

The segmentation process of somites can be subdivided into at least two phases. The first one is the patterning of the unsegmented PSM and the rostrocaudal compartmentalization within a presumptive somite. The second one is morphological segmentation, including intersomitic boundary formation. Molecular events in the first phase occur in the PSM, whereas those in the second phase occur mainly at the level anterior to the PSM. In accordance with the transition from the first to the second phase, the gene expression characteristic in the first phase is terminated. However, in *ripply1*-deficient embryos, this termination was disturbed. The gene expression characteristic in the first phase still remained even at the axial level anterior to the PSM, indicating that *ripply1* was required for this termination.

This means that the gene expression characteristic in the first phase requires a "specific" molecular apparatus for its termination. Thus, we suppose that some biological significance would exist to terminate actively the first phase-specific gene expression. Since *ripply1*-deficient embryos exhibited no distinct somite boundary, we can suppose that termination of the first phase-specific gene expression, which is performed by a molecular apparatus including Ripply1, may be a prerequisite for morphological segmentation. One possible way to test this hypothesis would be to analyze misexpression of genes involved in the first phase in somites. Injection of *mesp-b* mRNA into zebrafish embryos results in the absence of somite boundaries as in *ripply1*-deficient embryos (Sawada et al., 2000), but the immediate cause of this defect should be defective rostrocaudal polarization in the anterior PSM. Therefore, a better test would be a precise experiment that would force the first phase-specific gene expression in somites without disturbing the first phase.

Another important aspect of this study, regarding this termination, is the independency of cell differentiation on segmental patterning. In *ripply1*-deficient embryos, in spite of improper termination of the segment patterning machinery, differentiation markers of the paraxial mesoderm cells, i.e., myosin heavy chain, *myod*, and *pax9*, were expressed. Thus, although the segmentation machinery was not properly transited from the PSM to somites, the program required for the differentiation of the paraxial mesoderm cells appears to have proceeded normally. In other words, the molecular program required for cell differentiation would appear to proceed independent of the segmentation patterning machinery in the development of somites.

Molecular Mechanism Underlying the Rostrocaudal Compartmentalization of a Somite

The rostrocaudal polarity within the presumptive segmental unit is established by interaction between Mesp transcription factors and the Notch signaling pathway. However, it has remained obscure whether the initially established polarity is stable, or if some specific factor is required for its maintenance. Our results indicate that *ripply1* was specifically required for this maintenance. In embryos deficient in *ripply1*, the rostral compartment-specific expression of *mesp-a*, *mesp-b*, and *deltaD* was observed within the presumptive segmental unit in the anterior PSM, although the expression level of *mesp-b* was upregulated. However, in these embryos, the expression of markers for the rostral (*fgf8* and *papc*) and caudal (*myod*) compartment expanded almost uniformly at the axial level, where somites should be formed in normal embryos, and this defect in the maintenance of the polarity could be a cause for the loss of the somite boundary.

One of the important points for understanding the mechanism of the rostrocaudal polarity formation is the relationship between the machinery for its initial establishment and that for its maintenance. The induction of *ripply1* expression was dependent on *fss/tbx24*, but not on *mesp-b* and Notch signaling. Rather, *mesp-b* and Notch signaling were involved in the compartmentalization of the *ripply1* expression. Thus, *mesp-b* and Notch signaling, which are involved in the establishment of the rostrocaudal polarity, do not directly induce the expression of *ripply1*; however, their function in polarity establishment does lead to the rostral compartmentalization of the *ripply1* expression in somites.

However, the mechanism governing how *ripply1* maintains the rostrocaudal polarity remains to be revealed. In the anterior PSM of normal embryos, *ripply1* was expressed initially in the segment-wide domain and was subsequently restricted to the rostral part of the presumptive segment. Thus, one possible explanation is that Ripply1 that is expressed segment-wide may function in the proper polarized expression of the segmentation genes. In contrast, we may also speculate that the rostrally restricted expression is required for the proper rostral expression of the segmentation genes and that defective gene repression in the rostral compartment in *ripply1*-defective embryos may lead to subsequent improper gene expression in the caudal compartment. Intriguingly, as Oates et al. (2005) have recently indicated, *fss/tbx24* is required for the expression of the rostral genes in a cell-autonomous manner, and this gene is also required for proper gene expression in the caudal compartment in a cell nonautonomous fashion. Thus, some genes activated by *fss/tbx24* in the rostral compartment appear to be involved in this indirect gene expression in the caudal compartment. Since the induction of *ripply1* expression was dependent on *fss/tbx24*, the rostrally restricted *ripply1* may function downstream of *fss/tbx24* for caudal gene expression, thus completing the rostrocaudal polarization.

On the other hand, maintenance of the rostrocaudal polarity within somites is also reported to be disrupted in mouse embryos deficient in *Paraxis* or *Tbx18* (Bussen et al., 2004; Johnson et al., 2001). Thus, examination of a possible interaction between *ripply1* and *Paraxis* or *Tbx18* might shed more light on the function of *ripply1* in the maintenance of the rostrocaudal polarity.

Function of Ripply1 in Transcriptional Repression at the Transition from the PSM to Somites

The gene expression characteristic of the patterning process of segmentation was not properly terminated in *ripply1*-deficient embryos. This defective termination occurred at the transcription level, at least in the case of *mesp-b* and *her1*, and the nascent transcripts of these genes were also observed at the presumptive somite level of these embryos. These results suggest that Ripply1 is a component of the machinery for transcriptional repression of these genes. A series of our results strongly supports this idea. First, Ripply1 proteins were localized in the nucleus and were able to bind to the transcriptional corepressor Groucho in a manner dependent on the highly conserved WRPW motif (Fisher et al., 1996; Paroush et al., 1994). Second, overexpressed *ripply1* repressed *mesp-b* expression WRPW motif dependently in the anterior PSM. Therefore, Ripply1 appears to act as a component of a transcriptional repressor complex in gene repression essential for proper transition of segmentation machinery from the PSM to somites. Further identification of its target genes and other interactive molecules should reveal the function of Ripply1 in this transcriptional repression.

Experimental Procedures

Fish

All studies on wild-type zebrafish were performed by using the TL2 inbred line (Kishimoto et al., 2004). The mutant strains used in this study were *after eight* (*aei*^{tr233}), *fused somites* (*fss*^{til}), and *mind bomb* (*mib*^{ta52b}) (Itoh et al., 2003; van Eeden et al., 1996).

Isolation of *ripply* Genes in Zebrafish and Mouse

A 470 bp cDNA fragment of zebrafish *ripply1* was isolated by an in situ hybridization screening. The missing 5′ region was obtained by using cDNA derived from zebrafish embryos at the 15- to 18-somite stages and a SMART RACE cDNA amplification kit (Clontech). Based on the amino acid sequence of Ripply1 protein, zebrafish *rippy2* and *ripply3* and mouse *Ripply1*, *Ripply2*, and *Ripply3* were identified in the genomic database. According to the sequence information, the corresponding cDNAs were isolated from zebrafish cDNA (15- to 18-somite stages) and mouse E11.5 cDNA by using the SMART RACE cDNA amplification kit.

Plasmid Construction

For pCS2+ripply1 and pCS2+MT-ripply1, the DNA fragment encoding the entire amino acid sequence of zebrafish *ripply1* was inserted into either the pCS2+ or pCS2+MT vector. For pCS2+ripply1ΔWRPW and pCS2+MT-ripply1ΔWRPW, the nucleotide sequence corresponding to the WRPW tetrapeptide was depleted from either pCS2+ripply1 or pCS2+MT-ripply1 by PCR. For pCS2+MT-ripply1Cterm, the nucleotide sequence corresponding to the region from the carboxyl terminus to the WRPW motif was inserted into the pCS2+MT vector. For pBSK-grg2, the entire amino acid sequence of zebrafish grg2 was obtained by PCR.

In Situ Hybridization and Immunohistochemistry

Whole-mount in situ hybridization of zebrafish embryos was carried out as described (Nikaido et al., 1997). For the *mesp-b* intron probe, an ~750 bp DNA fragment corresponding to the second intron of the *mesp-b* genomic region was isolated by PCR and used as a template for an antisense probe. For in situ hybridization with the intron probe, embryos, briefly fixed with 4% paraformaldehyde, were hybridized at 50°C and then stained with successive changes of the staining buffer. Whole-mount in situ hybridization of mouse embryos was performed as reported earlier (Yoshikawa et al., 1997), and in situ hybridization on sections was carried out as previously described (Ohbayashi et al., 2002).

Immunostaining of zebrafish embryos was performed with an anti-chicken myosin heavy chain antibody, MF-20. For the visualization of myc-tagged Ripply1 protein, cultured Cos7 cells were transfected with the corresponding expression vector by using Fugene6 (Roche). After 24 hr of incubation, the cells were fixed for 30 min in 4% paraformaldehyde in PBS, and were then reacted with an anti-myc monoclonal antibody, 9E10 (SantaCruz). Fluorescence was detected from Alexa488-conjugated anti-mouse IgG antibody (Molecular Probe), and nuclei were counterstained with DAPI.

Antisense MO and mRNA Synthesis

The sequences of MOs used in this study were: *ripply1* MO1, 5′-CATCGTCACTGTGTTTTTCGTTTTG-3′; 5mis-*ripply1* MO1, 5′-CtTCcTCAgTGTcTTTTTCcTTTTG-3′; *ripply1* MO2, 5′-GCGCTGGTCGAGAATAGTCCGGCGG-3′; 5mis-*ripply1* MO2, 5′-GCcCTcGTCcAGAATAGTCaGGaGG-3′; *mesp-b* MO, 5′-TCGGTTCTTGCTTGAGGTTTGCATG-3′. *ripply1* MO1 and 5mis-*ripply1* MO1 were commonly used for all experiments, except when indicated otherwise. Capped mRNA was transcribed from linearized pCS2+ripply1 or pCS2+ripply1ΔWRPW by use of an mMessage mMachine (Ambion).

GST Pull-Down Assay

To generate GST-Ripply1 and GST-Ripply1ΔWRPW proteins, we inserted the DNA fragment corresponding to the amino acid sequence of ripply1 or that lacking the WRPW tetrapeptides in-frame into the pGEX4T-3 vector (Amersham), respectively. GST fusion proteins were induced by 0.5 mM IPTG, and they were purified by using glutathione Sepharose 4B (Amersham). For synthesis of in vitro-labeled Grg2 protein, 1 μg pBSK-Grg2 containing the entire amino acid sequence of zebrafish grg2 was added to a TNT Quick coupled Transcription/Translation Systems (Promega) in the presence of [^{35}S]-labeled methionine (Amersham). A total of 10 μl in vitro translation products was mixed with 1 ml 2% BSA in PBS(−), and the mixture was then incubated with 5 μg of either GST, GST-Ripply1, or GST-Ripply1ΔWRPW for 60 min at 4°C. After extensive washing, the bound proteins were separated in a 10% SDS-polyacrylamide gel.

Manipulation of Zebrafish Embryos

Transplantation of blastomeres at the blastula stage and the detection of labeled donor cells were performed essentially as described (Kane and Kishimoto, 2002). DAPT treatment was performed as described (Geling et al., 2002).

Supplemental Data

Supplemental Data including two figures and two tables are available at http://www.developmentalcell.com/cgi/content/full/9/6/735/DC1/.

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Accession Numbers

Nucleotide and amino acid sequences are registered with the Following accession numbers: zebrafish *ripply1*, AB212219; zebrafish *ripply2*, AB212220; zebrafish *ripply3*, AB212221; mouse *Ripply1*, AB212222; mouse *Ripply2*, AB212223.